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Fitness of antimicrobial-resistant Campylobacter and Salmonella

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Campylobacter and *Salmonella* are the most commonly reported bacterial causes of human foodborne infections, and increasing proportions of these pathogens become resistant to medically important antimicrobial agents, imposing a burden on public health. Acquisition of resistance to antibiotics affects the adaptation and evolution of *Salmonella* and *Campylobacter* in various environments. Many resistance-conferring mutations entail a biological fitness cost, while others (e.g. fluoroquinolone resistance in *Campylobacter*) have no cost or even enhanced fitness. In *Salmonella*, the fitness disadvantage due to antimicrobial resistance can be restored by acquired compensatory mutations, which occur both in vitro and in vivo. The compensated or even enhanced fitness associated with antibiotic resistance may facilitate the spread and persistence of antimicrobial-resistant *Salmonella* and *Campylobacter* in the absence of selection pressure, creating a significant barrier for controlling antibiotic-resistant foodborne pathogens.

Keywords
Antimicrobial resistance, Fitness, Campylobacter, Salmonella, Adaptation, Fluoroquinolone

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Forum on antimicrobial resistance

Fitness of antimicrobial-resistant *Campylobacter* and *Salmonella*

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Abstract

Campylobacter and *Salmonella* are the most commonly reported bacterial causes of human foodborne infections, and increasing proportions of these pathogens become resistant to medically important antimicrobial agents, imposing a burden on public health. Acquisition of resistance to antibiotics affects the adaptation and evolution of *Salmonella* and *Campylobacter* in various environments. Many resistance-conferring mutations entail a biological fitness cost, while others (e.g. fluoroquinolone resistance in *Campylobacter*) have no cost or even enhanced fitness. In *Salmonella*, the fitness disadvantage due to antimicrobial resistance can be restored by acquired compensatory mutations, which occur both in vitro and in vivo. The compensated or even enhanced fitness associated with antibiotic resistance may facilitate the spread and persistence of antimicrobial-resistant *Salmonella* and *Campylobacter* in the absence of selection pressure, creating a significant barrier for controlling antibiotic-resistant foodborne pathogens.

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1. Introduction

In general, bacterial resistance to antimicrobials occurs via either mutations in chromosomal loci or acquisition of horizontally transferred mobile genetic elements such as plasmids, phages, transposons and integrons [1–3]. The majority of the metabolic elements targeted by antimicrobials (e.g. the ribosome, DNA gyrase, RNA polymerase, and cell wall, etc.) are essential for bacterial growth. Therefore, genetic changes (mutations) in these genes that are associated with antibiotic resistance often have deleterious effects on the vital physiological processes in microorganisms [1–3]. As a result, acquisition of drug resistance, particularly the resistance mediated by chromosomal mutations, may incur a fitness cost in bacteria in the absence of antibiotic selection pressure [4–9]. Resistance acquired by horizontal gene transfer is thought to confer a competitive growth disadvantage to the cell by imposing additional energy expenditures that are needed to copy and transcribe plasmid replicons. 'Fitness cost' is defined as reduced growth and persistence of resistant bacteria in the host and environment or increased clearance from infected host and reduced transmission between hosts, and is typically measured via laboratory experiments in culture media or animal models [4,7,10]. However, compensatory mutations that partly or fully ameliorate the fitness cost associated with acquisition of antimicrobial resistance frequently occur in bacteria [5,7,11,12]. In addition, some resistance-conferring mutations bear 'low-cost' or 'no-cost' or even enhanced fitness [8,13–23]. As a result of both no-cost/low-cost mutations and compensatory evolution, antimicrobial-resistant bacteria are able to stabilize and persist in environments even in the absence of antibiotic usage [3,6,17–19,24,25].

Among bacterial pathogens, *Campylobacter* and *Salmonella* are the leading causes of human foodborne illnesses in the developed countries. Both *Campylobacter* and *Salmonella* are primarily intestinal organisms of a wide range of animals

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and are commonly present in the environment of food-producing animals [26,27]. In the last decades, *Campylobacter* and *Salmonella* have developed resistance to many clinically important antimicrobials, including fluoroquinolones (FQ) [28–32]. Although a large body of scientific information is available on the prevalence of antimicrobial resistance and its associated mechanisms in *Salmonella* and *Campylobacter*, many aspects related to persistence and dissemination of the antibiotic-resistant pathogens remain uncertain. Their transmission and spread are not only affected by the environmental and host factors, but also are influenced by the relative fitness of the drug-resistant organisms in the absence of selection pressure. In this paper we will review the recent literature on the fitness changes associated with antimicrobial resistance in *Campylobacter* and *Salmonella* and discuss the impact of the fitness changes on the control of the foodborne pathogens.

2. Fitness of antibiotic-resistant *Salmonella*

Many studies have been performed to determine the effect of resistance to streptomycin, fusidic acid, nalidixic acid, and rifampicin on the biological fitness of *Salmonella* [13,33–36]. A general finding from these studies is that the resistance mutations, at least initially, usually confer a fitness cost on *Salmonella* since these mutants were outcompeted by their wild-type strains in both culture media and animal models [6,7,13,35]. For example, fusidic acid resistance conferred by mutations in the *fusA* gene (encoding elongation factor G [EF-G]) and streptomycin resistance mediated by mutations in the *rpsL* gene (encoding ribosomal protein S12) resulted in reduced growth (in artificial growth medium and mice) of *Salmonella* due to a decrease in the rate of protein synthesis [34,35]. Further study indicated that the basal level (during steady-state growth) and the induced level (during starvation) of ppGpp, a global regulator for gene expression, were altered in fusidic acid-resistant *Salmonella* mutants, leading to an abnormal growth rate and cell size [37]. Since ppGpp positively regulates *rpoS*, fusidic acid-resistant *Salmonella* mutants also showed reduced ability to produce RpoS in the stationary phase [38]. RpoS is a global regulator for general stress response and an important virulence factor in *Salmonella*; and the reduced induction of RpoS in the fusidic acid-resistant mutants contributed to their low fitness in vivo [38]. In addition, fusidic acid-resistant *Salmonella* mutants were hypersensitive to oxidative stress, which was shown to be due to the reduced transcription of *hemA*, encoding an enzyme involved in the biosynthesis of heme [39]. Since heme is a cofactor for catalase, the reduced level of heme in the fusidic acid-resistant mutants impairs the function of catalases, resulting in hypersensitivity to oxidative stress [39].

The degree of fitness cost varies dramatically among different types of mutations that are associated with resistance to various antibiotics [13,35]. Although the majority of the mutations associated with resistance to streptomycin, fusidic acid, nalidixic acid, and rifampicin incur a fitness loss in mice, some of them did not show apparent growth disadvantage. For example, a streptomycin-resistant mutant carrying a mutation that alters AAA codon at position 42 to AGA in the *rpsL* gene (K42T change in the corresponding protein) was fully virulent in mice [13]. In addition, the initial fitness cost of antimicrobial resistance mutations can be partly or fully restored by compensatory mutations without loss of resistance in *Salmonella* [2,13,35]. The extent to which fitness was restored without loss of resistance varied dramatically and seemed to be dependent on the specific resistance phenotype. For resistance to streptomycin, nalidixic acid, and to a lesser degree rifampicin, most of the compensated mutants restored fitness in mice and in laboratory medium to wild-type or near wild-type levels, and some of the evolved mutants exhibited fitness qualities superior to that of the wild-type strains [13,35]. On the other hand, the relative fitness of the majority of fusidic acid-resistant *Salmonella* isolates in mice (but not in the culture medium) was substantially decreased even after compensatory evolution took place [35].

The types of compensatory mutations that restore fitness can be influenced by the environment in which the compensatory mutations are formed and selected. It was shown by Bjorkman et al. [35] that all streptomycin-resistant *Salmonella* isolates that were compensated in vitro accumulated extragenic compensatory mutations in the *rpsD* and *rpsE* genes, whereas when compensated in mice, they all contained intragenic suppressor mutations in the *rpsL* gene. On the other hand, for fusidic acid resistance, 26 of the 28 compensated mutants obtained in culture media had intragenic suppressor mutations in the *fusA* gene, and the other two mutants had true reversion to the susceptible genotype. In mice, the majority (14/25) of the compensated mutants were true revertants and the rest (11/25) of them were compensated by intragenic suppressor mutations. Both the true revertants and compensated mutants fully restored the fitness in the culture medium. However, only the true revertants were fully fit in mice, with the majority of the compensated mutants showing substantially reduced fitness in the mouse model. The different spectra of fusidic acid resistance compensatory mutations were thus likely caused by differences either in the rate of mutation formation or in the selective environment [35]. Because compensatory mutations can occur in multiple gene targets, the mutation rate for compensation is higher than that for reversion. Thus, compensatory mutations are usually more common than true reversion [7].

Compensatory mutations are deleterious to the evolved organisms when they are separated from the resistance-conferring mutations. For example, most extragenic compensatory mutations of streptomycin resistance alone, when separated from the resistance-conferring mutations in the compensated mutants or when introduced into the antibiotic susceptible wild-type background, incur a fitness cost on *Salmonella* [13,34,35]. From the view point of evolution, compensatory mutations must co-exist with resistance-associated mutations for fitness restoration. Thus, once a resistance-conferring mutation is acquired by pathogens, it may be difficult to revert it if compensatory mutations have already occurred, because the reversion to susceptibility without concomitant reverting of compensatory mutations will make the pathogen unfit [6].

How compensatory mutations restore the fitness deficit of antibiotic-resistant *Salmonella* is still not well understood in the majority of the cases. Fusidic acid inhibits protein synthesis rate by binding to EF-G·GDP and blocking its release from the ribosome [36]. Resistance to fusidic acid in *Salmonella* is mediated by mutation in *fusA*, the gene encoding EF-G. The resistance-associated mutations in EF-G decrease the rate of nucleotide exchange (GDP-to-GTP) and reduce the rate of protein synthesis [37]. In one study, 18 different types of intragenic compensatory mutations in EF-G were observed, correcting the fitness deficit fully or partly, probably by promoting the transition of EF-G from a GDP-binding state to a GTP-binding state [36]. Streptomycin binds to the ribosome and inhibits the elongation step in protein synthesis. Bacterial resistance to streptomycin is often mediated by mutations in ribosomal protein S12 encoded by *rpsL*. In streptomycin-resistant *Salmonella* due to restrictive mutations (increasing proofreading and decreasing the rate of protein synthesis) in S12, 35 different compensatory mutations that partly restored the fitness were identified in S4, S5, S12, and L19 [33]. Many of the compensatory mutations occurred in the interface between S4 and S5, and some of the mutations replaced the charged residues at the interface [33]. These changes presumably decrease translation fidelity and offset the over-increased rate of proofreading occurring in streptomycin-resistant mutants, thereby restoring the normal rate of protein synthesis [33].

A few reports documented the effect of resistance to clinically relevant antibiotics on the fitness of *Salmonella*. Highly fluoroquinolone-resistant mutants of *Salmonella* Typhimurium selected in vitro exhibited drastically altered growth characteristic on solid media (smaller colony size) [40]. Further experiments indicated that FQ-resistant (FQ^R) mutants selected in vitro or in vivo (chicken) varied dramatically in the level of resistance to FQ and the growth characteristics in culture medium and in chickens in the absence of FQ antimicrobials [41]. The in vitro selected mutants were highly resistant to FQs, showed significantly reduced growth rate in culture medium, and could not colonize chickens. In contrast, the in vivo selected resistant isolates exhibited intermediate susceptibility to FQs, had normal growth in liquid medium (slow growth on solid medium), and were able to colonize chickens at the extent comparable to or lower than that of the wild-type strains. After in vitro or in vivo passage in the absence of antibiotic selective pressure, partial restoration of the fitness was obtained without loss of the *gyrA* mutations [41]. Chromosomally encoded AmpC beta-lactamases have not been reported in *Salmonella* [9,11,42]. Artificial introduction of *ampC* from *Enterobacter cloacae* into *Salmonella enterica* serotype Typhimurium reduced its growth rate in culture media and its invasiveness to MDCK cells [42]. However, when *ampC* is co-transferred with *ampR*, which encodes a repressor for AmpC, the fitness cost associated with *ampC* production was eliminated, suggesting that the overexpression of *ampC* was deleterious to *Salmonella*. Recently, Hossain et al. [11] assessed the fitness of a clinical isolate of *Salmonella* shown to produce several beta-lactamases including a plasmid-encoded bla_{CMY-7} AmpC beta-lactamase. Both the clinical isolate and a *Salmonella* transconjugant bearing the bla_{CMY-7} plasmid were fully competent in culture medium and invasion of cultured mammalian cells, while a *Salmonella* transformant expressing the cloned bla_{CMY-7} alone was deficient in both growth rate and invasion of mammalian cells. This finding suggested that the fitness cost associated with high-level production of AmpC beta-lactamases in *Salmonella* may be compensated by plasmid-encoded functions [11]. The recent emergence of *Salmonella* populations resistant to extended-spectrum cephalosporins in clinical setting and animal reservoirs [32,43–45] further suggests that *Salmonella* has evolved mechanisms to compensate the loss of fitness associated with resistance to beta-lactams.

Resistance-conferring mutations as well as compensatory mutations occur frequently in bacteria exhibiting a mutator phenotype [46]. However, a permanent mutator phenotype accumulates more deleterious mutations than non-mutators and often shows a fitness disadvantage. As indicated by Chopra et al., the fitness disadvantage may be compensated by the high bacterial cell densities and the adoption of transient mutator status [46]. Mutators can be found in the natural populations of *Salmonella* and presence of mutator strains is considered a significant factor in the evolution of antibiotic-resistant *Salmonella* [47,48].

Salmonella infections caused by antibiotic-resistant isolates have been associated with increased risk of invasive illness, hospitalization and longer duration of illness compared with infections due to susceptible isolates [44,49–51]. It is difficult to ascertain if this seemingly increased severity of drug-resistant *Salmonella* infections is related to increased virulence of resistant isolates, or poor response to antibiotic treatment. Carriage of both resistance determinants and virulence genes on the same mobile element may enable some drug-resistant *Salmonella* isolates to be more virulent than drug susceptible strains [43,52,53]. A particular concern is with multi-resistant *Salmonella* Typhimurium definitive type 104 (mrDT104). mrDT104 has disseminated globally in humans and animals [29,32,54] and is associated with increased severity of clinical illnesses [43,50,51,55]. However, experimental systems failed to demonstrate enhanced virulence in mrDT104 isolates [56]. The effect of drug resistance determinants on the dissemination and virulence of mrDT104 is still questionable, and the genetic traits that are responsible for the virulence and global dissemination of mrDT104 remain to be elucidated [54].

3. Fitness of antibiotic-resistant *Campylobacter*

Unlike *Salmonella*, limited information is known about the effect of antimicrobial resistance on the ecological fitness of *Campylobacter*. Recently Luo et al. examined the persistence and biological fitness of FQ^R *Campylobacter* in chicken, a natural host for *Campylobacter*, in the absence of antibiotic selection pressure by using clonally related isolates and genetically defined mutants [23]. Several interesting findings were obtained from this study. When monoinoculated into the chickens, FQ^R *Campylobacter* was able to colonize and persist in chickens as

efficiently as the FQ-susceptible (FQ^S) strains in the absence of antibiotic usage. The prolonged colonization in vivo did not result in the reversion or loss of the specific resistance-conferring mutation (C257T, leading to a Thr86Ile substitution in the GyrA subunit) in *gyrA*. Surprisingly, when coinoculated into chickens, the FQ^R *Campylobacter* outcompeted the FQ^S parent strain and quickly became the dominant population in the chicken host. When a series of clonally related FQ^R and FQ^S isolates derived from chickens [57] were used in pairwise competition in chickens, the FQ^R *Campylobacter* outcompeted the majority of FQ^S strains. Notably, the fitness change in FQ^R *Campylobacter* isolates could not be explained by compensatory mutations because no mutations other than the resistance-conferring C257T mutation were detected in the *gyrA* and *gyrB* genes of the FQ^R strains. By using isogenic mutants generated via natural transformation, the fitness change was directly linked to the C257T mutation in *gyrA*, which confers high-level resistance to FQ antimicrobials in *Campylobacter* [23].

How the *gyrA* mutation affects *Campylobacter* fitness is still unknown. Since DNA gyrase is important for DNA supercoiling, the resistance-conferring mutation (C257T) in *gyrA* may affect the supercoiling activity of the mutant enzyme and consequently modulate the gene expression in *Campylobacter*. This possibility is being examined in the laboratory systems (Zhang, unpublished study). One interesting observation by Luo et al. [23] is that the same C257T mutation in *gyrA* can also incur a fitness cost in a small number of isolates. This finding suggests that when the C257T mutation is introduced to different genetic backgrounds, it can either enhance or reduce the fitness of FQ^R *Campylobacter*. How the *gyrA* mutation interacts with other genes in modulating *Campylobacter* fitness remains to be examined in future studies.

To determine the physiological traits that are associated with the enhanced fitness, FQ^R isolates were compared with the isogenic FQ^S strains using various in vitro assays [23]. When separately cultured in antibiotic-free laboratory media, FQ^R *Campylobacter* did not show any growth defect or enhancement compared to clonally related or isogenic FQ^S isolates, in agreement with an early observation made with a spontaneous FQ^R mutant selected in vitro [58]. The motility (an important factor in chicken colonization) of FQ^R *Campylobacter* was comparable with that of FQ^S strains. In addition, the expression level of the multidrug efflux pump CmeABC, which is essential for *Campylobacter* colonization in intestinal tract by mediating bile resistance [59], was also the same between the FQ^R and FQ^S strains. These observations indicated that the fitness change associated with FQ resistance cannot be explained by the phenotypes measured using the in vitro assays. In vivo studies may be required to define the physiological basis responsible for the fitness change of FQ^R *Campylobacter* in chickens.

The laboratory observations made with FQ^R *Campylobacter* are supported by findings from two epidemiological studies [60,61]. A recent work by Price et al. revealed that FQ^R *Campylobacter* was still prevalent in chickens from farms that had not used FQ antimicrobials for at least 1 year, indicating that the FQ^R *Campylobacter* was able to persist in the farms even in the absence of FQ usage [60]. Similarly, Pedersen and Wedderkopp found that FQ^R *Campylobacter* still existed in farms that had stopped using FQ for 4 years. PFGE analysis of the isolates indicated that some FQ^R *Campylobacter* clones were able to persist for several rotations on the farms in the absence of FQ usage, suggesting that these resistant clones were ecologically competent in the farm environments [61]. These observations suggest that FQ^R *Campylobacter,* once evolved, may continue to persist in chicken flocks regardless of the use of FQ antimicrobials.

Several studies reported that the human patients infected by FQ^R *Campylobacter* had prolonged diarrhea, increased risk of invasive illness, and hospitalization compared with patients infected with FQ^S *Campylobacter* [12,62,63]. Whether the increased clinical severity was related to enhanced virulence of FQ^R *Campylobacter* or due to treatment failure is unknown. In developed countries, human-to-human transmission of *Campylobacter* is rare and campylobacteriosis is primarily a foodborne disease. The fitness change associated with FQ resistance would impact the persistence and prevalence of FQ^R *Campylobacter* in animal reservoirs and eventually affect the health of the human hosts.

Analysis of the genomic sequences [64,65] revealed that *Campylobacter* lacks many genes encoding components of DNA repair systems that are present in other bacteria. In particular, genes encoding the MutH and MutL components of the methyl-directed mismatch repair are absent in *Campylobacter* spp. This system seems to be also absent in the related *Helicobacter pylori*, explaining the high mutation rates observed in this bacterium [66]. *Campylobacter* also lacks the LexA repressor (involved in SOS response), the *umuCD* genes (UV-induced mutagenesis), SbcB exodeoxyribonuclease I (recombination repair), the *vsr* gene (very short patch repair), the *ada* gene (adaptative response to alkylating agents), and the *phr* gene (encoding photolyase enabling repair of pyrimidine dimers by photoreactivation). These defects in the repair systems may contribute to the ability of *Campylobacter* to mutate and acquire antibiotic resistance traits.

Although many *Campylobacter* strains are resistant to non-FQ antibiotics, the effect of acquiring resistance to non-FQ antimicrobials on *Campylobacter* fitness is unknown and remains to be investigated in future studies. Gibreel et al. [67] recently demonstrated the stability of the macrolide resistance-conferring mutations (except the A2074G mutation in 23S rRNA) by repetitive subcultures. However, it is unknown if macrolide-resistant *Campylobacter* can compete with macrolide-susceptible strains in the absence of macrolide antibiotics. In the related bacterium *H. pylori*, clarithromycin resistance appears to incur a biological cost [66]. A recent work by Luangtongkum et al. (T. Luangtongkum, T.Y. Morishita, L. Martin, I. Choi, and Q. Zhang. Abstract no. A55, 13th International Workshop on *Campylobacter*, *Helicobacter*, and Related Organisms, Gold Coast, Australia, September 4–8, 2005) revealed the high prevalence and dynamic change in tetracycline-resistant *Campylobacter* strains on organic poultry farms, where no antibiotics were used for the production. It was found that tetracycline-resistant

Campylobacter co-existed with or even replaced pre-existing tetracycline-susceptible strains in the chicken flocks in the absence of antibiotic usage. The majority of the tetracycline-resistant strains carried the *tet*(*O*) plasmid. In another study by Avrain et al. [68] it was shown that the horizontal transfer of the *tet*(*O*) gene could occur rapidly between *Campylobacter jejuni* strains in the intestinal tract of chickens in the absence of antibiotic selection pressure. Tetracycline-resistant *Campylobacter* strains were also frequently isolated from organic dairy farms [69]. Together, these findings suggest that tetracycline-resistant *Campylobacter* has evolutionally adapted to the animal production environment and may continue to persist on farms regardless of the use of tetracycline antibiotics.

4. Concluding remarks

It is apparent that acquisition of antimicrobial resistance affects the adaptation and evolution of *Salmonella* and *Campylobacter* in various environments. Some resistance-conferring mutations incur a fitness cost, while others have low or no cost or even enhanced fitness in the absence of selection pressure. Compensatory mutations that ameliorate fitness cost frequently occur in *Salmonella*. These observations may help explain the persistence of resistance phenotypes in modern isolates to antimicrobials that are used rarely or not at all. The types of compensatory mutations and the level of restoration of fitness vary with the antibiotics to which the resistance-conferring mutations are developed and the environment in which the compensatory mutations are formed and selected. With *Campylobacter*, both laboratory studies and epidemiological surveys indicated that many FQ^R mutants are as competent as or even more fit than FQ^S strains, which may partly explain the increasing prevalence of FQ^R *Campylobacter* on a global scale. Since antibiotic-resistant *Salmonella* and *Campylobacter* may still retain fitness in the absence of antibiotic usage, reduction or discontinuation of antimicrobial use in the farm environment alone may not necessarily result in, at least in the short term, a decrease in the frequency of antibiotic-resistant isolates. Understanding how resistance-conferring mutations affect the adaptation and evolution of microbial pathogens will facilitate the development of means to predict and prevent transmission of antibiotic-resistant foodborne pathogens.

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